

1 USE OF STREPTOMYCES HYALUROLYTICUS ENZYME IN OPHTHALMIC

2 TREATMENTS

3 Field of the Invention

4 The instant invention relates to the field of enzyme therapy, specifically the use of high
5 purity hyaluronidase from *Streptomyces hyalurolyticus* for the treatment of ophthalmic disorders.

6 Description of the Prior Art

7 Hyaluronidase is a well-known enzyme with a variety of applications. Hyaluronidase is
8 naturally occurring and breaks down various mucopolysaccharides such as hyaluronic acid and
9 the chondroitin sulfates. It is found as a family of enzymes and comes in both monomeric and
10 oligomeric forms. The enzyme presents itself over a broad range of molecular weights and
11 specificities. It is commercially available from a variety of sources including calf or sheep testes,
12 helminthes, leeches, bee venom, snake venom and bacterial sources such as *Streptomyces* and
13 *Streptococcal* species. Various suppliers including REANAL Company, Serva and Sigma
14 Chemical Company produce and distribute hyaluronidase from mammalian sources. A common
15 form of the enzyme, known as Wydase®, is available from Wyeth-Ayerst Laboratories, Inc.
16 Philadelphia, PA. Wydase® is a preparation of purified bovine testicular hyaluronidase that
17 contains various impurities including proteolytic enzymes.

18 Wydase® is currently used to prepare the eye for treatment of a variety of ophthalmic
19 disorders. Hyaluronic acid, one substrate for hyaluronidase, is a polysaccharide widely found in
20 the extracellular connective tissue of animals. It is also the primary constituent of the vitreous of
21 the eye. Diabetic retinopathy, trauma to the eye and other disorders of the eye can cause rupture
22 of the retinal blood vessels or leakage from these vessels, which results in blood entering the
23 vitreous humor. The vitreous humor can then become cloudy after such intravitreal hemorrhage.

1 In certain cases, intravitreal hemorrhage can lead to further complications that require quick
2 diagnosis and surgical repair. Such complications can include retinal detachment and formation
3 of fibrous tissue at the site of hemorrhage. Vision may become reduced or even totally impaired
4 in this event, thus it is imperative to treat the hemorrhage and remove the opacity that contributes
5 to such risk. Bovine testicular hyaluronidase is injected into the vitreous humor to remove
6 hemorrhagic blood and clarify the vitreous humor prior to transvitreal viewing of the retina or
7 further treatment of the detached portion. Karageozian et al in US Patent 5,866,120 and US
8 Patent 6,039,943 describe the use of bovine testicular hyaluronidase for this purpose. These
9 patents and the methods described therein are incorporated by reference as though reproduced in
10 their entirety.

11 In another use, hyaluronidase is used to soften the cornea of the eye in a form of
12 orthokeratology or vision correction. This treatment is commonly referred to as enzyme
13 orthokeratology. Harris in US Patent 5,788,957 and US Patent 5,626,865 describes the use of
14 bovine hyaluronidase to soften the cornea prior to refractive correction. This patent and the
15 methods described are incorporated by reference as though reproduced in its entirety.

16 Hyaluronidase has also been used to reduce intraocular pressure in the eyes of glaucoma
17 patients through degradation of the hyaluronic acid within the vitreous humor. This application is
18 described in US patent 4,820,516 to Sawyer and Edwards.

19 In another version of ophthalmic enzymology, hyaluronidase is added extraocularly as a
20 means for spreading local anesthesia more effectively through tissue prior to surgical
21 interventions. This use as a "spreading agent" also applies to the application of drugs for the
22 treatment of ocular diseases.

1 Finally, in an optometric use of hyaluronidase, Fedorov et al in US Patent 6,037,144
2 describe the use of hyaluronidase for preparing artificial lenses. The method comprises steps of
3 providing collagen-containing cattle basal membrane, incubating the membrane in a mixture of
4 pepsin, hyaluronidase and acetic acid and separating the collagen from the mixture. After
5 conditioning, the collagen can be mixed with monomers and polymerized to produce strong,
6 elastic intraocular lenses and contact lenses that are highly biocompatible and gas permeable.

7 A form of the hyaluronidase from the bacterium *Streptomyces hyalurolyticus* was
8 described in US patent number 3,728,223 to Kaneko et al, which is incorporated by reference as
9 though reproduced in its entirety. The *S. hyalurolyticus* enzyme is currently in use in the health
10 food and animal feed industries but has not been considered applicable to the medical field
11 because of its susceptibility to proteolytic inactivation.

12 It is clear that hyaluronidase has multiple uses for treating disorders of the eye, yet the
13 commonly used source of surgical hyaluronidase, bovine testes, represents an inefficient source
14 due to its activity and specificity, contamination by unwanted molecules such as proteases and
15 by the complicated process required to purify the enzyme for clinical use. In an analysis of the
16 Wydase® hyaluronidase for contaminants, protease activity ranging from 0.0216 units per mL to
17 0.0593 units per mL was found depending on the lot tested. This contamination was found in
18 enzyme lots that had hyaluronidase activities ranging from 2.44 turbidity-reducing units (TRU)
19 per mL to 4.82 TRU per mL.

20 Summary of the Invention

21 The invention provides for the use of an alternative source of hyaluronidase, purified
22 from *Streptomyces hyalurolyticus*, for the treatment of ophthalmic disorders. The *S.*
23 *hyalurolyticus* hyaluronidase requires fewer steps to achieve higher purity and has specificity for

1 hyaluronic acid and not other glycosaminoglycans. The enzyme can be effectively used in
2 medical techniques when contaminating protease is not a concern such as treatments of the eye.
3 These advantages make this bacterially derived hyaluronidase more effective and less expensive
4 for ophthalmic applications.

5 The enzyme is prepared from the bacterium, *Streptomyces hyalurolyticus*. Growth of
6 bacteria in large-scale culture is inexpensive and preparation of the enzyme requires fewer steps
7 than the mammalian forms. The bacterial preparation provides a form of the enzyme with
8 significantly less contamination from protease, mammalian virus, immunogens and other
9 unwanted particles than other commercially available sources. Use of the *S. hyalurolyticus*
10 enzyme in the eye and particularly in the vitreous humor to prepare intravitreal hemorrhage for
11 treatment is ideal because of its high activity levels relative to other sources of the enzyme and
12 because of its specificity for hyaluronic acid. Because of this high relative activity, a lower
13 concentration of the enzyme is required and the cost to the user is reduced. Furthermore, protease
14 levels in the mammalian eye and vitreous humor are extremely low and do not interfere with the
15 activity of the bacterial enzyme, making the enzyme an ideal candidate for ophthalmic and
16 optometric uses in humans. The hyaluronidase obtained from the *S. hyalurolyticus* source
17 provides substantial economic and medical advantages over enzyme obtained from mammalian
18 sources.

19 Detailed Description of the Invention

20 Hyaluronidase is a naturally occurring enzyme that breaks down mucopolysaccharides.
21 The enzyme is derived from a variety of sources but is primarily derived from calf testes for
22 biomedical use. Other sources include sheep testes, helminthes, leeches, bee venom, snake
23 venom and bacteria such as *Streptomyces* and *Streptococcal* species. Depending on the source,

1 the enzyme's substrate specificity varies. Although the preferred source of the enzyme, bovine
2 testes, is currently used in some medical applications, the purification sequence necessary to use
3 the enzyme is laborious and not always effective at producing high purity or high activity
4 enzyme.

5 Production of a stable hyaluronidase from a bacterial source was attempted as early as the
6 1970's, and it was reported that a specific strain of the genus *Streptomyces*, *S. hyalurolyticus*
7 produced hyaluronidase (Biochimica Biophysica Acta. 198 (1970) 607-609). Hyaluronidase
8 from *S. hyalurolyticus* has the advantage of being specific for hyaluronic acid alone and not the
9 other glycosaminoglycans. This enzyme carries out an elimination reaction that results in the
10 production of double bonds at the nonreducing end of hyaluronic acid. However, this new
11 hyaluronidase was determined to be unstable due to inactivation by protease, and accordingly
12 was not considered sufficient for use as a therapeutic enzyme. In particular, contamination by
13 protease or use in systems where proteases are inherent rendered the enzyme inactive and made
14 its medical use prohibitive. Recently, an improved method for purifying hyaluronidase from *S.*
15 *hyalurolyticus* was achieved whereby the enzyme activity levels far exceeded the nominal
16 contamination by protease. Additional purification steps are possible to eliminate all protease
17 contaminants and provide a high activity, high purity source of hyaluronidase for medical
18 procedures.

19 Purification methods for hyaluronidases are well developed and widely distributed in the
20 literature. They include such common techniques as extraction, precipitation, centrifugation,
21 ultrafiltration and chromatography. Production of hyaluronidase from bacterial sources has now
22 been found to be faster and less costly than isolation from mammalian testes and contaminating
23 macromolecules can be more easily avoided in a bacterial system than in tissue extraction.

1 In an exemplary purification scheme, hyaluronidase produced by *S. hyalurolyticus* is
2 harvested from bacteria grown in a suitable culture medium such as that described in US patent
3 3,728,223. The techniques used in the various purification steps are well known in the art and are
4 described in the '223 patent as well as various references in enzymology and protein purification
5 including *Guide to Protein Purification: Methods in Enzymology*, M.P. Deutscher editor,
6 Academic Press 1997. The supernatant from the growth medium is filtered to remove cells and
7 an ultrafiltration step is performed to further remove pigments and other particulate matter. The
8 proteins within the filtrate are precipitated with a first precipitant and filtered then precipitated
9 with a second precipitant and filtered. After dissolution of the final precipitate and filtration with
10 Sephadex, two ion exchange chromatography steps are performed to isolate the hyaluronidase.
11 Dialysis of the isolate is performed to remove salts followed by membrane filtration and freeze-
12 drying. A variety of tests, including polyacrylamide gel electrophoresis, enzyme linked
13 immunosorbent assays and substrate-gel assays are known in the art to determine purity and
14 activity of the hyaluronidase.

15 The use of hyaluronidase from *S. hyalurolyticus* for medical purposes is still subject to
16 inhibition or deactivation due to the variety of proteolytic enzymes inherent to biological
17 systems. For ophthalmologic uses, particularly those described above, inactivation of the
18 hyaluronidase is minimal due to the small amount of protease found in the vitreous humor. The
19 vitreous humor is greater than 99% water. Less than 1% of the vitreous humor is comprised of
20 macromolecules, particularly substances similar to those found in albumin. Proteolytic enzymes
21 are found in minimal concentrations; these are insufficient to affect the activity of the *S.*
22 *hyalurolyticus* hyaluronidase. Thus a clear but unexpected advantage exists in using this easily
23 purified and highly active source of hyaluronidase in the eye.

1 A contaminant analysis of hyaluronidase purified from *S. hyalurolyticus* (Amano Enzyme
2 Company, Nagoya, Japan) demonstrates significantly reduced protease activities in the range of
3 0.00316 units per mL to 0.0188 units per mL and substantially higher hyaluronidase activities
4 (152 to 218 TRU per mL) than found in the bovine enzyme. This means that less *S.*
5 *hyalurolyticus* enzyme is necessary per treatment. Although the hyaluronidase obtained from *S.*
6 *hyalurolyticus* is reported to be susceptible to protease inactivation, less contaminating protease
7 means that the enzyme is more stable for ophthalmic use. These advantages of easier and higher
8 yield purification, higher enzyme activity and use in a system that is essentially free from
9 inactivating proteases, make this source of hyaluronidase a better candidate for ophthalmologic
10 uses.

11 In a preferred use of hyaluronidase purified from *S. hyalurolyticus*, the enzyme is
12 formulated in an injectable thimerosal-free preparation. The enzyme, prepared as a lyophilized
13 powder for injection, is reconstituted in a balanced salt solution, commonly made up of 0.6 to
14 0.7% sodium chloride, 0.07 to 0.08% potassium chloride, 0.04 to 0.05% calcium chloride
15 dihydrate, 0.02 to 0.04% magnesium chloride hexahydrate, 0.3 to 0.4% sodium acetate
16 trihydrate, 0.1 to 0.2% sodium citrate dihydrate and sodium hydride/ hydrochloric acid to adjust
17 the pH to 6.0, then sterile water qs to 100%.

18 This reconstituted enzyme is injected intravitreally once at six to sixteen days prior to
19 transvitreal viewing of the retina. Dosing is in the range of 50 to 250 IU of hyaluronidase with
20 the preferred dosage being 50 to 100 IU. Clearing of hemorrhage from the vitreous humor occurs
21 over the described times and such clearing is significantly faster than that which would have
22 occurred without hyaluronidase treatment.

1 In an extraocular use, a single application of the formulation described above can be
2 added topically to the eye fifteen minutes to twelve hours prior to administration of anesthesia.
3 This improves the penetration and efficacy of anesthetics prior to ophthalmic surgery.

4 In another use, *S. hyalurolyticus* hyaluronidase is used to soften the cornea of the eye
5 prior to enzyme orthokeratology. The technique modifies or degrades the hyaluronic acid-
6 derived structural component of the cornea, allowing the cornea to become softer and more
7 pliable. The cornea can then be reshaped using a contact lens or other means. In this technique,
8 the lyophilized form of *S. hyalurolyticus* hyaluronidase is reconstituted in the carrier solution
9 described above or another pharmaceutically acceptable carrier. Anesthetics such as
10 proparacaine hydrochloride can be included in the solution to anesthetize the cornea. A dose of
11 hyaluronidase appropriate for softening the cornea, typically between 100 and 1500 IU per
12 milligram of substrate, is applied topically and the cornea is allowed to soften prior to reshaping
13 and refractive correction.

14 In an optometric use of *S. hyalurolyticus* hyaluronidase, the enzyme is used in preparing
15 artificial lenses. Following the disclosure of the '144 patent, the method comprises incubating
16 collagen-containing cattle basal membrane in a mixture of pepsin, hyaluronidase and acetic acid
17 and separating the collagen from the mixture. After conditioning, the collagen is mixed with
18 monomers and polymerized to produce strong, elastic intraocular lenses and contact lenses that
19 are highly biocompatible and gas permeable.

20 The preceding examples are provided for descriptive purposes solely and are not meant to
21 limit the embodiments of the invention. Other formulations and uses of the *S. hyalurolyticus*
22 hyaluronidase enzyme will become apparent to those of ordinary skill in the art.